

1 **High current optogenetic channels for stimulation and inhibition of** 2 **primary rat cortical neurons**

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9 **Abstract**

10 ChR2-XXL and GtACR1 are currently the cation and anion ends of the optogenetic single
11 channel current range. These were used in primary rat cortical neurons *in vitro* to manipulate
12 neuronal firing patterns. ChR2-XXL provides high cation currents via elevated light sensitivity
13 and a prolonged open state. Stimulating ChR2-XXL expressing putative presynaptic neurons
14 induced neurotransmission. Moreover, stable depolarisation block could be generated in single
15 neurons using ChR2-XXL, proving that ChR2-XXL is a promising candidate for *in vivo*
16 applications of optogenetics, for example to treat peripheral neuropathic pain. We also
17 addressed an anion channelrhodopsin (GtACR1) for the next generation of optogenetic neuronal
18 inhibition in primary rat cortical neurons. GtACR1's light-gated chloride conduction was verified
19 in primary neurons and the efficient photoinhibition of action potentials, including spontaneous
20 activity, was shown. Our data also implies that the chloride concentration in neurons decreases
21 during neural development. In both cases, we find surprising applications of these high current
22 channels. For ChR2-XXL inhibition and stimulation are possible, while for GtACR1 the role of Cl⁻
23 during neural development becomes a new optogenetic target.

24 **Introduction**

25 The past discovery of Channelrhodopsin (ChR) 1/2^{1,2} enabled a broad range of novel
26 neurophysiological experiments using optogenetics^{3,4}. The corresponding optogenetic
27 applications are commonly limited by the functional expression in a particular model system or
28 the rhodopsin's features, like distinct specificity for particular ions. To reach a high level of
29 expression and the corresponding number of functionally produced rhodopsins, the
30 supplementation of the chromophore all-trans-vitamin-a-aldehyde (retinal) has proved to be

31 helpful⁵ and under some conditions the supplementation is even obligatory³. Recently a ChR2
32 mutant called ChR2-XXL was published to overcome this limitation of optogenetics with strikingly
33 high expression and slow closing kinetics enabling high photocurrents even under low light and
34 retinal conditions⁶. Similar to its precursor, ChR2-XXL is a relatively unselective proton and
35 cation channel enabling neuronal firing during illumination with blue light (**Fig. 1a**). The past
36 optogenetic approaches to inhibit neuronal activity have been based on light-driven pumps and
37 are thus less efficient. Recent studies describe the discovery of Anion Channelrhodopsins
38 (ACRs)⁷ as an alternative. Within this class, GtACR1 seems to have a relatively strict anion
39 conductance and offers the possibility of efficient light gated neuronal silencing (**Fig. 1a**). ChR2-
40 XXL already proved to be a powerful optogenetic tool in *Drosophila Melanogaster*. Here we
41 further tested the two high current optogenetic channels (ChR2-XXL and GtACR1) in a
42 mammalian system (primary rat cortical neurons) to propose what protocols are needed to
43 control neural activity without any side effects on the cells. Both high ion current light-driven tools
44 offer a next generation of neurophysiological optogenetics. Here we highlight consequent
45 applications and set the field for advanced optogenetics.

46 Peripheral neuropathic pain originates in the peripheral nerve fibres, most commonly in the arms
47 and legs^{8,9}. Scientists of neural engineering are seeking improved approaches that can be used
48 to block pain fibres selectively and reversibly to alleviate episodes of debilitating chronic pain. An
49 optogenetic approach^{2,10,11} offers elegant ways to achieve this aim. Especially, ChR2-XXL⁶,
50 which can give rise to the largest photocurrents of all published ChRs and mutants with an
51 increased light sensitivity more than 10,000-fold over wild-type ChR2 in *Drosophila* larvae. This
52 results in gating by diffuse, ambient light and enables an efficient threshold surpassing
53 depolarisation for non-invasive neuronal control. Moreover it is likely that a stable depolarisation
54 block can be induced by photostimulating ChR2-XXL expressing neurons due to its large
55 photocurrent¹², inactivating the voltage-gated sodium channels¹³, or activation of calcium-
56 activated K⁺ channels, resulting in a block of neuronal over-excitation¹⁴.

57 Optogenetic studies of neurotransmission will play an important role in the fields of behaviour as
58 well as neuronal disorders. Moreover, ChR2-XXL with its characteristic long open-state⁶ likely
59 allows future investigations of synaptic plasticity *in vivo*. Therefore, investigating
60 neurotransmission by stimulating ChR2-XXL expressing cultured neurons will build a
61 fundamental basis.

62 Channelrhodopsin-2 and variants thereof² were generated and characterised to become a
63 toolbox for activating neural activity in response to light and are widely applied in neuroscience

64 ^{3,4,15–17}. In contrast, inhibitory tools are less frequent in the literature and unequally effective for
65 inhibiting neurons. However, recently discovered naturally occurring anion channelrhodopsins
66 (ACRs) of the alga *Guillardia theta* might overcome these limitations and imbalance ⁷ by
67 exclusive anion conductance. ACRs seem to be able to silence neuronal activity with high
68 sensitivity and efficiency, ⁷ presumably due to their strict conduction of anions such as Cl⁻.
69 Complete exclusion of protons and cations, results in their effective hyperpolarisation of the
70 neural membrane, and usefulness in decoding brain functions.

71 Here, we present possible applications of high ion current optogenetics by expressing ChR2-
72 XXL or GtACR1 in primary rat cortical neurons. We introduced optogenetic investigations of
73 neurotransmission with ChR2-XXL in primary rat cortical neurons and moreover we investigated
74 whether a stable depolarisation block can be successfully induced in ChR2-XXL expressing
75 neurons. Furthermore, the function of GtACR1 was validated in primary cortical neurons by
76 successfully achieving photoinhibition of spiking induced by a pulsed current as well as inhibition
77 of spontaneous activity. Our findings will strongly facilitate and broaden these tools to investigate
78 how the inhibitory or excitatory neurons control the behaviour. Aside from the efficiency of ChR2-
79 XXL and GtACR1 in standard applications, these additional uses show that there are more areas
80 of optogenetics to be developed using high current channels. These two high current
81 optogenetic channels (ChR2-XXL and GtACR1) will provide a sufficient toolbox for optical
82 control to decode neural circuits and understand basic brain function ^{18,19}.

83 **Results**

84 **GtACR1 or ChR2-XXL in *Xenopus laevis* oocytes**

85 We validated GtACR1 by generating a GtACR1::YFP construct and injecting the cRNA into
86 *Xenopus laevis* (*X.l.*) oocytes for TEVC recordings. High photocurrents were already detected 16
87 hours post injection (hpi) of 5 ng/oocyte cRNA with saturating 530 nm light pulses (**Fig. 1b**).
88 Surprisingly, no obvious YFP signal was observed by fluorescence microscopy at this time,
89 indicating a low GtACR1 expression level and relatively high single channel conductance. ChR2-
90 XXL's increased photocurrent is associated with a stronger expression level ^{6,20}. To further
91 validate these observations, photocurrents and the fluorescence of oocytes expressing GtACR1
92 or ChR2-XXL were measured at distinct time points after injection of corresponding cRNA.
93 Photocurrents of GtACR1 reached about 30±3% at 18 hpi, 42±10% at 42 hpi of the maximum
94 current recorded after the normal expression time of 66 hpi. In contrast, ChR2-XXL
95 photocurrents were 5±1% at 18 hpi, 30±8% at 42 hpi and 47±7% at 66 hpi relative to the
96 photocurrents observed in case of GtACR1 (**Fig. S 1a**). However, the fluorescence of oocytes

97 expressing ChR2-XXL was at least 2-times higher than of those expressing GtACR1 at all three
98 time points though the amount of injected ChR2-XXL cRNA was four times higher (**Fig. S 1b, c**).
99 This manifests that ChR2-XXL's single channel conductance is strongly reduced relative to
100 GtACR1.

101 As for ChRs, the functional expression of GtACR1 should depend on retinal binding ²¹. In
102 *Xenopus laevis* oocytes, the GtACR1 photocurrent at 16 hpi was ~3 times higher in culture
103 buffer supplemented with 1 μ M all-trans-retinal than without (**Fig. 1c**), which is similar to the
104 change in current observed for ChR2-XXL⁶. Since GtACR1::YFP fluorescence was not obvious
105 at 16 hpi, the YFP signal at 64 hpi was investigated but no obvious difference could be seen (**Fig.**
106 **1d**), showing that the high single channel conductance produced a distinguishable photocurrent
107 at such a low expression level that a difference in YFP signal is not detectable. Interestingly we
108 also observed that the GtACR1 fluorescence accumulated at distinct spots (**Fig. S 2b**).

109 **Neurotransmission induced with ChR2-XXL**

110 To functionally validate the powerful optogenetic tool ChR2-XXL⁶ in primary rat cortical neurons,
111 the DNA of ChR2-XXL was delivered into neurons using electrofection on the day of preparation.
112 Very clear YFP fluorescence was detected on DIV13 (**Fig. 2a**) indicating ChR2-XXL expression.
113 Fluorescence was generally homogeneous over the soma and neurites indicating an even
114 distribution of the channel.

115 High photocurrents and light sensitivity make possible easy monitoring of neurotransmission,
116 therefore we applied ChR2-XXL in primary rat cortical neurons and customised a protocol for
117 neurotransmission studies (**Fig. 2b**). For this, neurons exhibiting fluorescence were selected as
118 putative presynaptic neurons. Neurons without fluorescence were assumed to be postsynaptic
119 neurons. A ChR2-XXL-expressing neuron (the putative presynaptic neuron) was photostimulated,
120 while a ChR2-XXL negative neuron (the putative postsynaptic neuron) was recorded using
121 whole cell patch clamp. In order to laser stimulate with high-fidelity, the distance between the
122 putative presynaptic and postsynaptic neurons was longer than 200 μ m but located in the same
123 0.15 mm² frame of imaging. All measurements were done in the dark to avoid activation of
124 ChR2-XXL by stray light. The principle is that neurotransmitter release from the ChR2-XXL
125 positive presynaptic neuron under the blue light stimulus could elicit a detectable postsynaptic
126 current (PSC) in the putative postsynaptic neuron. If excitatory, this can make the postsynaptic
127 neuron surpass its threshold and fire action potentials.

128 Based on the above described protocol, inward currents were successfully evoked in putative
129 postsynaptic neurons from DIV8 or DIV15 when illuminating the putative presynaptic neurons
130 (ChR2-XXL positive) (**Fig. 2c**). Moreover, interestingly, a relatively high number of action
131 potentials were recorded at the putative postsynaptic neurons (**Fig. 2d**), especially during long-
132 duration photostimulation. Compared to laser stimulations of just 1 second, the postsynaptic
133 spiking continued significantly longer when the laser stimulated for 60 seconds (**Fig. 2e**). These
134 data indicate that neurotransmission could be shaped in primary rat cortical neurons using
135 ChR2-XXL.

136 **Depolarisation block induced by ChR2-XXL**

137 Because ChR2-XXL could generate the largest photocurrents of all ChRs published, and
138 showed light sensitivity more than 10,000-fold over wild-type ChR2 in *Drosophila* larvae⁶, we
139 investigated whether ChR2-XXL could induce a stable depolarisation block when exposed to
140 weak white light for a few minutes. In order to achieve depolarisation block, all neurons were
141 illuminated 10 minutes with weak white light (<8.5 nW/mm² from a xenon bulb). Subsequently,
142 patch clamp recordings were made. After white light exposure, ChR2-XXL positive neurons
143 needed more injected current than control neurons to hold the membrane potential at -70 mV
144 regardless of DIV (**Fig. 3a**). The reason for this should be a change in the resting potential of
145 these ChR2-XXL positive neurons caused by the continued currents through ChR2-XXL. Next,
146 we measured the resting potential using patch clamp in current-clamp mode, with no current
147 applied. The resting potentials of most of ChR2-XXL positive neurons were higher than those of
148 control cells across different culture days (DIV7 to DIV14) (**Fig. 3b**). From our recordings with
149 primary rat cortical neurons, a depolarisation to about -50 mV evoked an action potential during
150 electrical stimulation. However, most of the ChR2-XXL positive neurons surpassed this threshold
151 after exposure to dim white light (**Fig. 3b**). Depolarisation block drives the membrane potential
152 over threshold and holds it there.

153 Subsequently we investigated whether the membrane potential could be depolarised in ChR2-
154 XXL positive neurons by laser stimulation after a depolarisation block. Together with a laser
155 stimulus, patch clamp was employed to characterise ChR2-XXL positive cortical neurons. All
156 representative voltage traces indicated two kinds of patterns (**Fig. 3c**). The first pattern showed
157 a declining membrane potential prior to laser stimulation due to exposure to the room lighting
158 while setting up the experiment, which demonstrated ChR2-XXL had been activated but was
159 gradually closing. This is consistent with ChR2-XXL's long open state and high light sensitivity⁶.
160 The second pattern was that depolarisation took place without action potentials during laser

161 stimulation of ChR2-XXL-expressing neurons, indicating that the voltage-gated channels were
162 still inactivated. Moreover, prolonged laser stimulation failed to elicit action potentials. These
163 data demonstrated that ChR2-XXL can be used to induce a stable state of depolarisation block.

164 A depolarisation block forces neurons into a refractory state without the ability to produce action
165 potentials due to inactive voltage-gated sodium channels¹³. Therefore, we designed an
166 approach using voltage clamp to measure how voltage-gated inward current was changed and
167 confirmed that it could be recovered in ChR2-XXL positive neurons after neurons were blocked.
168 To recover blocked neurons (**Fig. 3d**), voltage was stepped from -70 mV to -20 mV by +10 mV
169 steps each held for 20 msec. Then a hyperpolarisation pulse was applied and those steps
170 repeated. Different hyperpolarisations were tested as in Bendahhou²². No inward current was
171 detected before or after hyperpolarisation at -130 mV for 5 secs in ChR2-XXL positive neurons,
172 whereas the amplitude of voltage-gated inward current was slightly increased after
173 hyperpolarisation in a control neuron (**Fig. 3e**). These data indicated that hyperpolarisation can
174 un-block a control neuron, but those particular conditions were not sufficient for un-blocking the
175 ChR2-XXL expressing neuron. Therefore we further decreased the hyperpolarizing pulse for
176 ChR2-XXL positive neurons. Finally, at -170 mV for 2 secs or -210 mV for 2 secs, a current
177 recovery was seen, whereas it never appeared at -150 mV for 2 secs (**Fig. 3f**). Moreover,
178 compared with the hyperpolarisation at -170 mV for 2 secs, voltage-gated current was slightly
179 increased after hyperpolarisation at -210 mV, but it was still lower than the voltage-gated current
180 in the control neurons. These data indicated that depolarisation block could be successfully
181 induced using ChR2-XXL and that it could be recovered by hyperpolarisation to -170 mV or -210
182 mV for 2 secs.

183 **Light-gated chloride conduction by GtACR1 during neural development**

184 Having successfully induced neurotransmission and a depolarisation block in primary rat cortical
185 neurons using a depolarizing tool, we aimed to apply the high current, light-gated, anion channel
186 GtACR1 to silence neuronal activity by hyperpolarisation. Light-gated proton pumps²³ or
187 modified ChRs with additional chloride conductance²⁴⁻²⁷ are rather inefficient compared to
188 optogenetic channels. GtACR1-YFP DNA was transfected into primary rat cortical neurons by
189 electrofection before seeding cells on PDL-coated coverslips. Very clear yellow fluorescence
190 was detected in the neurons transfected with GtACR1 on DIV16 (**Fig. 4a**). However,
191 fluorescence was intermittent along the neural membrane including the membrane of axons or
192 dendrites. These data indicated GtACR1 expression in the membrane of neurons.

193 Next, the function of GtACR1 was investigated by electrophysiology and light induced opening.
194 GtACR1 can be activated with a saturating blue light pulse (**Fig. S 2a**), so here blue laser was
195 employed to investigate GtACR1 in primary cortical neurons. The membrane of cortical neurons
196 was hyperpolarised by blue laser stimulation for 0.25 milliseconds (**Fig. S 4a**), hyperpolarisation
197 was induced by GtACR1 with a mean rise time (τ_R) of 1.23 ± 0.14 milliseconds and a mean
198 decay time (τ_D) of 126.03 ± 19.43 milliseconds (**Fig. S 4b**). This is similar to slow switching
199 components observed in GtACR1 by Sineschchekov et al [33]. Voltage step sequences applied
200 during and 1.5 s after illumination exhibited lower currents at each holding potential. Moreover,
201 voltage-gated peak current was eliminated by laser stimulation (see: inset in **Fig. S 5a**). At early
202 stages of neuronal development (DIV6 and DIV8), the IE relationship showed a linear
203 relationship (**Fig. 4b**). However, at later, more mature states of neurons (DIV14, DIV17 and
204 DIV20) a rectification appeared. Therefore, we investigated whether the synaptic inputs in the
205 mature neural network resulted in this rectification. The later mature state of neurons (DIV14,
206 DIV16 and DIV20) still showed similar rectification behaviour on stationary photocurrents (**Fig. S**
207 **6**). Therefore, the rectification was not associated with synaptic inputs in the neuronal network.
208 Next, we compared the voltage-dependent currents during photostimulation and dark-periods.
209 Currents during photostimulation fit to a linear shape (**Fig. 4c** and **Fig. S 5c**), whereas these
210 currents under dark showed a rectification behaviour (**Fig. S 5b** and **d**), which contributed to the
211 age-dependent rectification behaviour of the stationary photocurrent (**Fig. 4b**). In a word, the
212 change in rectification is not associated with the GtACR1 channels, but the state of the neurons
213 in which they are expressed.

214 Furthermore, the reversal potential for GtACR1 during neural development (from DIV6 to DIV20)
215 showed two kinds of trends (**Fig. 4d**). For younger neurons from DIV6 to DIV8, the reversal
216 potential declined from $-66.4 \text{ mV} \pm 3.4 \text{ mV}$ to $-72.2 \text{ mV} \pm 2.1 \text{ mV}$. The reversal potential was
217 then steady through DIV17 at about -72 mV ($p = 0.958$ for comparisons of DIV8 to DIV14 and p
218 $= 0.8417$ for DIV8 to DIV17). Then at DIV20, it further declined to $-78.4 \text{ mV} \pm 1.6 \text{ mV}$ ($p = 0.032$,
219 DIV17 vs. DIV20). These data imply the chloride concentration in neurons was changing during
220 neural development.

221 **Photoinhibition of spiking by GtACR1**

222 Having successfully validated the light-gated chloride conductivity of GtACR1 in primary rat
223 cortical neurons, we investigated how GtACR1 mediated neuronal membrane potential
224 alterations. First, we addressed photoinhibition of spiking induced by pulsed currents. To induce
225 spiking, 10 millisecond long 300 pA depolarizing electric pulses were applied at 20 Hz to primary

226 rat cortical neurons expressing GtACR1 via the patch clamp pipette in whole cell mode. For a
227 typical GtACR1-expressing neuron on DIV8, illumination successfully inhibited induced neuronal
228 spiking compared to current injections performed in the dark (black trace vs. green trace, see:
229 **Fig. 5a**). Also, the same results were obtained at DIV14 (**Fig. 5b**) when neurons are embedded
230 in a neural network and should also be receiving synaptic inputs. Because the chloride
231 concentration in neurons was different during neural development (**Fig. 4d**), we wanted to verify
232 whether GtACR1 could inhibit neural activity at different culture days. Therefore we tested 4-7
233 neurons at each different culture day (from DIV6 to DIV20). Interestingly, all spikings induced by
234 pulsed current were successfully inhibited by light, regardless of the neuronal age and
235 corresponding chloride concentrations. These data indicate that the large currents generated by
236 GtACR1 overcome neural stimulation even when chloride concentration reduces flow through
237 the channel.

238 Subsequently, we further addressed spontaneous neural activity in mature networks. Neurons
239 expressing GtACR1 at DIV 20 were selected for whole cell patch clamp recordings. In response
240 to photostimulation, spontaneous activity of all neurons measured ($n=10$ neurons expressing
241 GtACR1 from DIV20) was successfully inhibited (**Fig. S 7**). Furthermore, spontaneous activity
242 was repeatedly suppressed with intervening return to spontaneous firing in the dark (**Fig. 5d**).
243 These data demonstrate GtACR1 could effectively and reversibly inhibit spontaneous neural
244 activity by illumination.

245 Furthermore, whether hyperpolarisation induced by GtACR1 affects the spiking after illumination
246 is still unknown. In order to address this open gap, during the recordings of GtACR1-expressing
247 neurons a continuous depolarizing injection of 100 pA was applied to generate continuous action
248 potentials. Simultaneously, laser pulses were applied to induce hyperpolarisation. The amplitude
249 of action potentials after light illumination was slightly increased (**Fig. 5c**). Demonstrating that
250 the hyperpolarisation by GtACR1 could recover the voltage-gated channels in neurons, resulting
251 in increasing the action potential's amplitude (**Fig. 5c**).

252 However, by multiple illuminations, the frequency of action potentials showed a decreasing
253 phenomenon. This may be due to after turning off the light inhibition the cells rebound and fire
254 too much, and too much firing triggers more slow calcium activated outward K^+ currents as a
255 response to over-excitation^{14,28}. Also, we tested whether the amplitude of hyperpolarisation by
256 GtACR1 is changed after multiple activations. The photocurrent evoked by the laser pulse
257 decreased to 75% after ~ 10 pulses (**Fig. S 8a and b**). Neural membrane potential was

258 hyperpolarised by multiple light pulses (**Fig. S 8c and d**) and interestingly, the hyperpolarisation
259 amplitude decreased following multiple pulses due to GtACR1's desensitisation property.

260 Discussion

261 The high photocurrents of GtACR1 and ChR2-XXL that have been shown here and previously in
262 X.l. oocytes is attributable to different mechanisms. High currents reached for GtACR1 with low
263 cRNA injection and expression levels below the fluorescence detection limit suggest higher
264 single channel conductance. Whereas, ChR2-XXL reached high currents by producing twice as
265 many channels, according to fluorescent tag intensity. The rapid establishment of detectable
266 GtACR1 currents in X.l. oocytes provides a platform for the further study of GtACR1 by site-
267 directed mutagenesis. The aim of these efforts will be to generate mutants with faster kinetics
268 and a red-shifted absorption spectrum.

269 ChR2-XXL activation was used to control non-expressing putative postsynaptic cells.
270 Interestingly, the spiking of putative postsynaptic neurons persisted after the end of a stimulus
271 (**Fig. 2e**) with a slow decline. This phenomenon reflected the long open-state of ChR2-XXL. In
272 addition, the amplitude of action potentials declined during the illumination (**Fig. 2d**). After laser
273 illumination and a short dark period, the amplitude of action potentials increased to the initial size
274 observed during spontaneous activity. This phenomenon implies that synaptic inputs elicited by
275 the large and sustained photocurrent of ChR2-XXL, had blocked the recovery of some sodium
276 channels from inactivation, resulting in reduced action potentials. Furthermore, the spiking in
277 these postsynaptic neurons showed an oscillating decline (**Fig. S 3**). We hypothesise that the
278 observed oscillating decline is associated with ChR2-XXL inducing rhythmic patterns of calcium
279 influx in the presynaptic neurons, which can trigger exocytosis of neurotransmitter-containing
280 synaptic vesicles via mechanisms shown previously²⁹. The photocurrent of ChR2-XXL shows a
281 prolonged decay⁶, which is presumably responsible for the decline of the spike rate.

282 With sustained current injection, neurons eventually enter a depolarisation block, which can
283 protect them from excessive spiking activity³⁰. Here, we have shown that ChR2-XXL can be
284 used to induce a depolarisation block (**Fig. 3c**) by exposing the neurons to a halogen lamp's
285 white light for 10 minutes. After this, all voltage traces (**Fig. 3c**) showed a decreased potential in
286 advance of the first laser photostimulation, indicating that ChR2-XXL channels were still not
287 completely closed. White light with low intensity is able to keep ChR2-XXL channels open,
288 resulting sustained depolarization. After 10 minutes exposure to white light, our results showed
289 that photostimulation failed to provoke action potentials (see: **Fig. 3c**). These data suggest that
290 because the peak current and sensitivity are so high, even using the tail of the absorption

291 spectrum, it may be possible to manipulate ChR2-XXL with longer wavelength light that is more
292 favoured for *in vivo* applications. Moreover, it was difficult to recover this state by
293 hyperpolarisation at lower holding voltages. The need for at least -170 mV hyperpolarisation to
294 re-set voltage gated channels suggests most natural processes would fail to overcome
295 manipulation by ChR2-XXL. Our results support ChR2-XXL as a potential tool to block highly
296 excitable neurons, as seen in some disorders like peripheral neuropathic pain.

297 To block neural firing by the more classical approach of hyperpolarization GtACR1 was
298 expressed in primary cortical neurons. Contrary to the expectation that older neurons would
299 have produced more protein and therefore have higher photoinduced currents, the response at
300 DIV8 was similar to DIV17 or DIV20 (**Fig. 4c**). It may be that the CMV promoter is only stable in
301 neurons of particular types³³, independent of the transgene, since during neuronal development
302 GtACR1 shows normalised currents with light that are not significantly different (**Fig. S 5c**). The
303 recombinant Adeno-Associated Virus 6 (rAAV6) with hSyn promoter system would eliminate this
304 issue in primary rat cortical neurons by utilizing a continually active neuron-specific promoter.
305 The use of rAAVs would furthermore increase the percent of neurons expressing GtACR1³⁴.
306 Another factor influencing the current over time may be the ECl changes that have been shown
307 to occur in the developing rodent brain³⁵. Figure 4d implies chloride concentration was
308 decreasing during neural development, consistent with other studies^{35,36}. It may therefore be
309 possible to use GtACR1 to manipulate chloride concentration during cell maturation to further
310 study these processes.

311 **Methods**

312 **Molecular biology**

313 The ChR2-XXL-YFP fragment was amplified through Polymerase Chain Reaction with Phusion
314 High-Fidelity DNA Polymerase (2 U/ μ L, # F-530S, Thermo Scientific, Germany) based on the
315 plasmid named pGEM ChR2-XXL-YFP. Primer pairs for its amplification are listed below (**Table**
316 **1**). For ChR2-XXL subcloning, the psc-hSyn-ChR2-XXL was generated using restriction digest-
317 based strategies³⁷, with the backbone produced from psc-hSyn-ChR2opt¹⁷ using double-digest
318 with *Hind* III and *Bam* HI.

Name	Sequence	Remark
XXL-Forward	5'- CCC AAGCTT GCC ACC ATG GAT TAT GGA GGC GCC CTG AGT	5' Enzyme site: Hind III Kozak sequence(GCCACC)
XXL-Reverse	5'- CG GGATCC T TAC TTG CCG GCG	3' Enzyme site: Bam HI

GCC GCT TTA CTT GT

319 **Table 1** Primer pair for amplification of ChR2-XXL-YFP

320 5' Enzyme site is marked in red colour, 3' Enzyme site is marked in blue colour. Kozak sequence
321 ³⁸ improving efficient initiation of translation is marked in black colour with bold font.

322 The GtACR1 sequence was synthesised by GeneArt Strings DNA Fragments (Life technologies,
323 Thermo Fisher Scientific) according to the publication ⁷, while the codon usage was optimised to
324 *Drosophila Melanogaster*. For *Xenopus* oocyte expression, the GtACR1 DNA was inserted into
325 the pGEMHE vector within N-terminal BamHI and C-terminal XhoI restriction sites; a YFP tag
326 was fused after the XhoI restriction site. For cortical neuron expression, the GtACR1-YFP
327 sequence was cut from pGEMHE-GtACR-YFP and inserted into the PBK CMV vector using N
328 terminal BamHI and C terminal HindIII restriction sites. The constructs were then confirmed by
329 sequencing (GATC, Konstanz).

330 **Maxi preparation of Plasmids**

331 A single colony was picked from a freshly streaked selective plate and used to inoculate a starter
332 culture of 5 ml LB medium containing the antibiotic Ampicillin (50 µg/mL). The starter culture was
333 incubated for approx. 8 h at 37°C with vigorous shaking (approx. 225 rpm). 200 ml (pBK-CMV-
334 GtACR1-YFP) or 500 ml (psc-hSyn-ChR2-XXL due to its low-copy) medium were inoculated with
335 250–500 µl of starter culture. Then cultures were grown at 37°C for 12–16 h with vigorous
336 shaking (approx. 225 rpm). To purify plasmids for electroporation, EndoFree Plasmid Maxi Kit
337 (#12362 Qiagen, Germany) was used following the protocol the kit provided. In the last step, the
338 DNA was dissolved in 200 µL of TE buffer. To determine the yield, DNA concentration was
339 determined by both the microvolume UV-Vis spectrophotometry (NanoDrop 1000, Thermo
340 Fisher Scientific, MA, USA) at 260 nm and quantitative analysis on an agarose gel after
341 electrophoresis.

342 **Primary Rat Cortical Neuron culture and Electrofection**

343 Primary cortical cultures were prepared as described previously ³⁹. Briefly, cortices from
344 embryonic day 18 (E18) Wistar rat brains were dissected and mechanically dissociated by
345 trituration with a fire-polished, silanized pasteur pipette in 1 ml Hank's balanced salt solution
346 without calcium or magnesium (HBSS-) (0.035% sodium bicarbonate, 1 mM pyruvate, 10 mM
347 HEPES, 20 mM glucose, pH 7.4). The cell suspension was diluted 1:2 in HBSS+ (with calcium
348 and magnesium) and non-dispersed tissue was allowed to settle for 3 min. The supernatant was

349 centrifuged for 2 min at 200 g. Amaxa Rat Neuron Nucleofector® Kit (Cat.No. VPG-1003) from
350 Lonza Cologne GmbH (Germany) was used to transfer circular, double stranded, plasmid DNA
351 directly into cells. Each electrofection experiment used two fresh cortices, dissociated and
352 collected as described above. After centrifugation for 2 min at 200 g, the cell pellet was carefully
353 resuspended in 200 µl room temperature Nucleofector® Solution from the Kit (163.6 µl of Rat
354 Neuron Nucleofector® Solution mixed with 36.4 µl of Supplement), to which was added 6 µg of
355 psc-hSyn-ChR2-XXL plasmid or 8.5 µg pBK-CMV-GtACR1-TYE plasmid. The cell/DNA
356 suspension was quickly transferred into a certified cuvette without producing bubbles. Using the
357 Nucleofector® Device (AAD-1001S, Lonza, Germany), program G-013 was applied to the cells.
358 800 µl of the pre-equilibrated RPMI medium (Gibco, Grand Island, NY, USA) with 1% FBS
359 (Gibco, Grand Island, NY, USA) and 0.5 mM L-glutamine (Gibco, Paisley, UK) were immediately
360 added to the cuvette. The cells were counted in a Neubauer counting chamber and plated in a
361 concentration of 50,000 cells per well (24-well-plate) on poly-D-lysine (PDL, 10 µg/ml) coated,
362 12 mm diameter coverslips in 500 µl supplemented RPMI medium per well. After 4 hours, the
363 medium was carefully replaced with 500 µl fresh supplemented Neurobasal medium (NB) per
364 well to remove cellular debris (supplemented Neurobasal medium (Gibco, Paisley, UK) with 1%
365 B-27 (Gibco, Grand Island, NY, USA), 0.5 mM L-glutamine (Gibco, Paisley, UK) and 50 µg/ml
366 gentamicin (Sigma, Steinheim, Germany)). Cells were kept at 37°C, 5% CO₂ and 100% humidity
367 in 500 µl NB. Every third day, half of the medium was exchanged.

368 This work except for GtACR1 experiments was carried out with the approval of the
369 Landesumweltamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen,
370 Recklinghausen, Germany, in accordance with §6 TierschG., §4 TSchG i.V. and §2 TierSchVerV.
371 GtACR1 experiments were carried out with the approval of the Landesumweltamt für Natur,
372 Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany, number 84-
373 02.04.2015.A173.

374 **Fluorescence Microscopy and quantification of homogenised oocytes**

375 To qualitatively monitor the expression of YFP-tagged ionotropic photoreceptors conventional
376 fluorescence microscopy was used. The YFP fluorescence of oocytes was detected by Leica
377 DMI8. Bright field settings for the inverted microscope were always 100 ms exposure and gain
378 1.5. Corresponding settings for detection of YFP fluorescence were always 1600 ms exposure
379 and gain 1.8.

380 To quantify the fluorescence intensity, *Xenopus* oocytes expressing the indicated constructs
381 were homogenised by pipetting and the resulting lysate was loaded into Nunc surface 96-well

382 plates and investigated using a corresponding fluorometer. The fluorescence emission was then
383 measured at 538 nm by a Fluoroskan Ascent microplate fluorometer with 485 nm excitation.

384 **Oocyte electrophysiology**

385 cRNA was generated with the AmpliCap-MaxT7 High Yield Message Maker Kit (Epicentre
386 Biotechnologies) using NheI-linearised pGEM-HE GtACR1 YFP plasmid. Oocytes were injected
387 with the indicated amount of cRNA and incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1
388 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES and 50 µg/ml Gentamycin, pH 7.4) containing 1 µM
389 ATR. Ringer's solution (110 mM NaCl, 5mM KCl, 2 mM BaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH
390 7.6) was used as standard buffer for Two electrode voltage-clamp recordings with the indicated
391 holding potential. 2s 473 nm blue laser and 530 nm green laser saturating light pulses were
392 used for illumination.

393 **Neuron electrophysiology**

394 Whole cell patch clamp experiments were performed using an EPC9 amplifier (HEKA Elektronik,
395 Lambrecht/Pfalz, Germany) controlled by the TIDA 5.05 software (HEKA Elektronik). The
396 recordings were performed in extracellular patch clamp buffer solution (mM: NaCl 120, KCl 3,
397 MgCl₂ 1, HEPES 10, CaCl₂ 2; pH 7.3). For the synaptic input blocking measurements, the
398 following (all from Tocris Bioscience, Bristol, UK) were added to the extracellular solution above:
399 D-AP5 (25 µM), NBQX (10 µM), SR 95531 hydrobromide (alternative name: Gabazine, 20 µM).
400 Patch pipettes were pulled from borosilicate glass capillaries (Sutter Instrument Co., Novato, CA,
401 USA) using a micropipette puller (P-2000, Sutter) to achieve a pipette resistance of 6-9 MOhm.
402 For ChR2-XXL measurements the pipettes were filled with intracellular patch solution (mM: NaCl
403 2, KCl 120, MgCl₂ 4, HEPES 5, EGTA 0.2, Mg-ATP 0.20, pH 7.3). For GtACR1 measurements,
404 an intracellular solution with low chloride concentration (mM: K₂SO₄ 67.5, MgCl₂ 2, adjusted to
405 248 mosm/kg with glucose) was used.

406 **Laser stimulation**

407 Optogenetic channel expressing neurons were stimulated with a 473 nm diode laser (Rapp
408 OptoElectronic GmbH, Hamburg, Germany), which was guided through an optical fibre and
409 reflected with a dichroic mirror (UGA-40, Rapp OptoElectronic GmbH) into the light path of a
410 Zeiss Axioscope microscope equipped with a x20 water immersion objective (Olympus,
411 UMPLFLN20xW). Different delay times (500 msec to 60 sec) and different laser pulse times
412 (ranging from 0.25 msec to 100 sec) were applied for light stimulation. Light intensity was 1.68
413 W/mm² for ChR2-XXL stimulation (laser spot diameter was about 24 µm); GtACR1 can also be

414 activated with a saturating blue light pulse (**Fig. S 2a**), light intensity was 0.21 W/mm² for
415 GtACR1 (laser spot diameter was about 61.2 μm).

416 **Data analysis**

417 Data were analysed in TIDA 5.240, Excel, Matlab, or Origin 9. The data are given as mean ±
418 standard error of the mean (neurons) or s.d. (oocytes). For ChR2-XXL data, the spikes whose
419 amplitudes were more than 35 mV were selected to determine the oscillation of firing frequency.
420 Each frequency point was the average value of 5 seconds window. For GtACR1 measurements,
421 stationary photocurrents of GtACR1 were measured for the last 50 ms of the illumination period,
422 and then were corrected by subtraction of the current values from the last 50 milliseconds
423 without light to adjust the baseline to zero. Reversal potentials were calculated by linear fit
424 between a measured positive and a measured negative stationary current to extract the I=0 pA
425 crossing point (here between two data points at voltage step of -60 mV or -80 mV and -70 mV).

426 **Data Availability**

427 The datasets generated during and/or analysed during the current study are available from the
428 corresponding author on reasonable request.

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430 The authors would like to thank B. Breuer for preparation of primary neurons.

431 **Conflict of Interest**

432 The authors declare no conflicts of interest.

433 **Author Contributions**

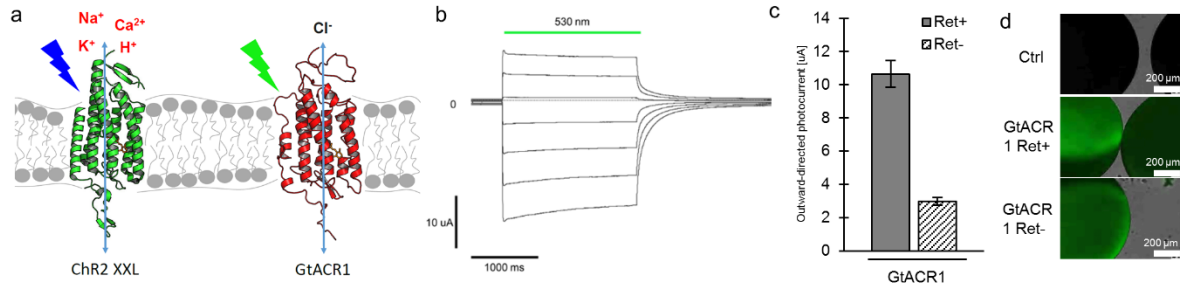
434 Conceptualisation: LJ, SG, AO. Data collection: LJ, EFJ, SG, WL. Data curation: SG, AO.
435 Formal analysis: LJ, EFJ, SG, VM. Supervision: SG, VM, AO. Visualisation: LJ, EFJ, VM, SG,
436 AO. All authors have contributed to and read the final version of the manuscript.

437 **References**

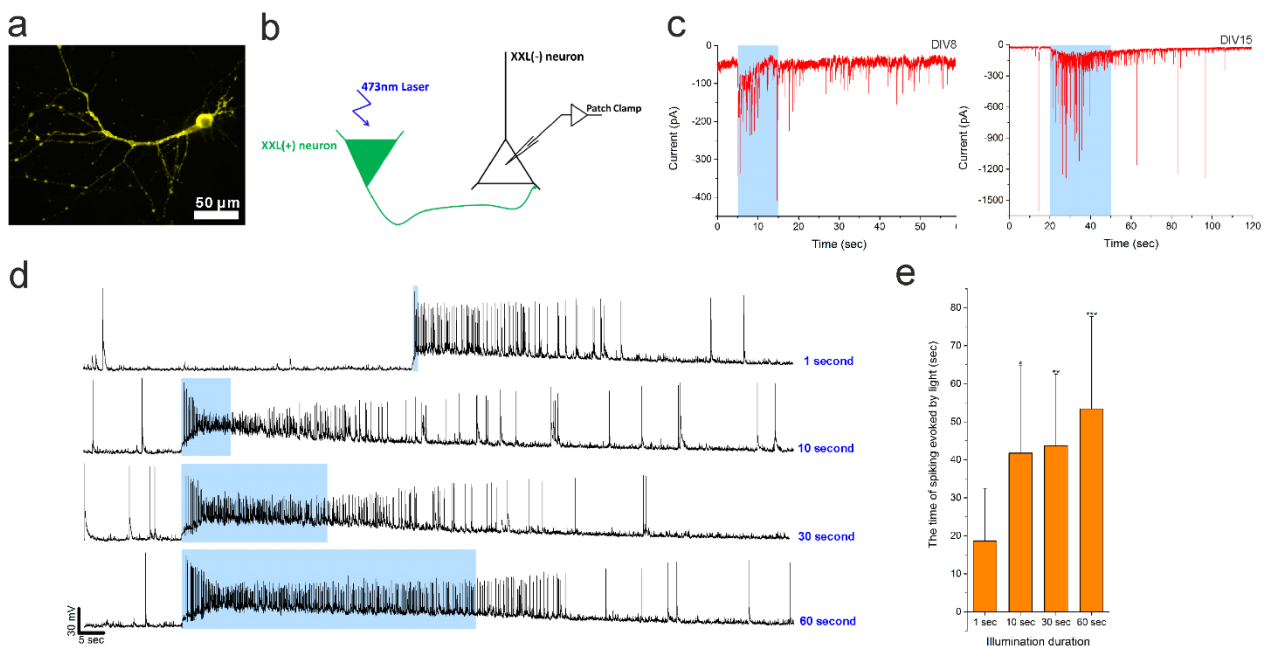
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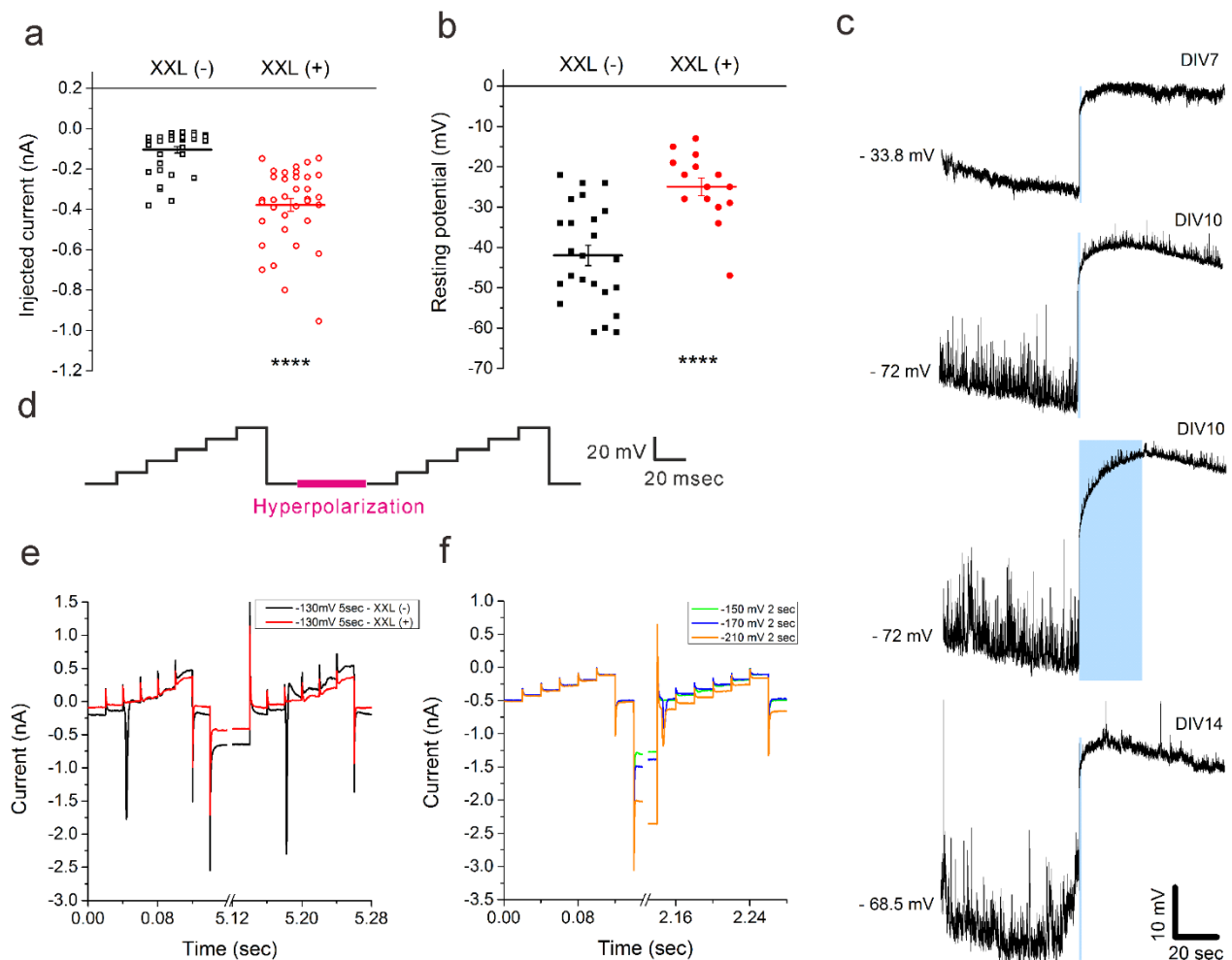
527
 528 **Figure 1 GtACR1 in *Xenopus laevis* oocytes** A) Schemes displaying the basic functional
 529 properties of ChR2-XXL (green) and GtACR1 (red). ChR2-XXL relatively unselectively conducts
 530 protons and cations, such as sodium, after irradiation with blue light (473 nm), whereas GtACR1
 531 conducts anions, such as chloride, after irradiation with green light (530 nm) under physiological
 532 conditions. b) Photocurrents generated by a selected GtACR1 expressing *Xenopus laevis* (X.I.)
 533 oocyte 16 hours after injection of 5 ng cRNA (16 hpi). A saturating 530 nm light pulse (2 s)
 534 induces current as membrane potential is changed in +20 mV steps from -100 mV to +20 mV. c)
 535 Mean stationary photocurrent at -100 mV of oocytes (X.I.) expressing GtACR1 (16 hpi) after
 536 incubation in ND96 solution supplemented with 1 μ M all-trans retinal (Ret+) or without (Ret-)
 537 (Ret+ $n=10$, Ret- $n=9$). d) Observed fluorescence of selected oocytes (X.I.) expressing the
 538 GtACR1::YFP construct 64 hpi and after incubation in Ret+ or Ret- ND96 (Ret+, Ret-).
 539 Electrophysiological recordings were done in ORI BaCl₂ solutions pH 7.6. Rhodopsins were
 540 activated using a laser with corresponding wavelength at saturating intensities (GtACR1 530 nm
 541 = 18.2 mW/mm²). Each statistical evaluation is showing an individual paired experiment. Error
 542 bars indicate s.d.



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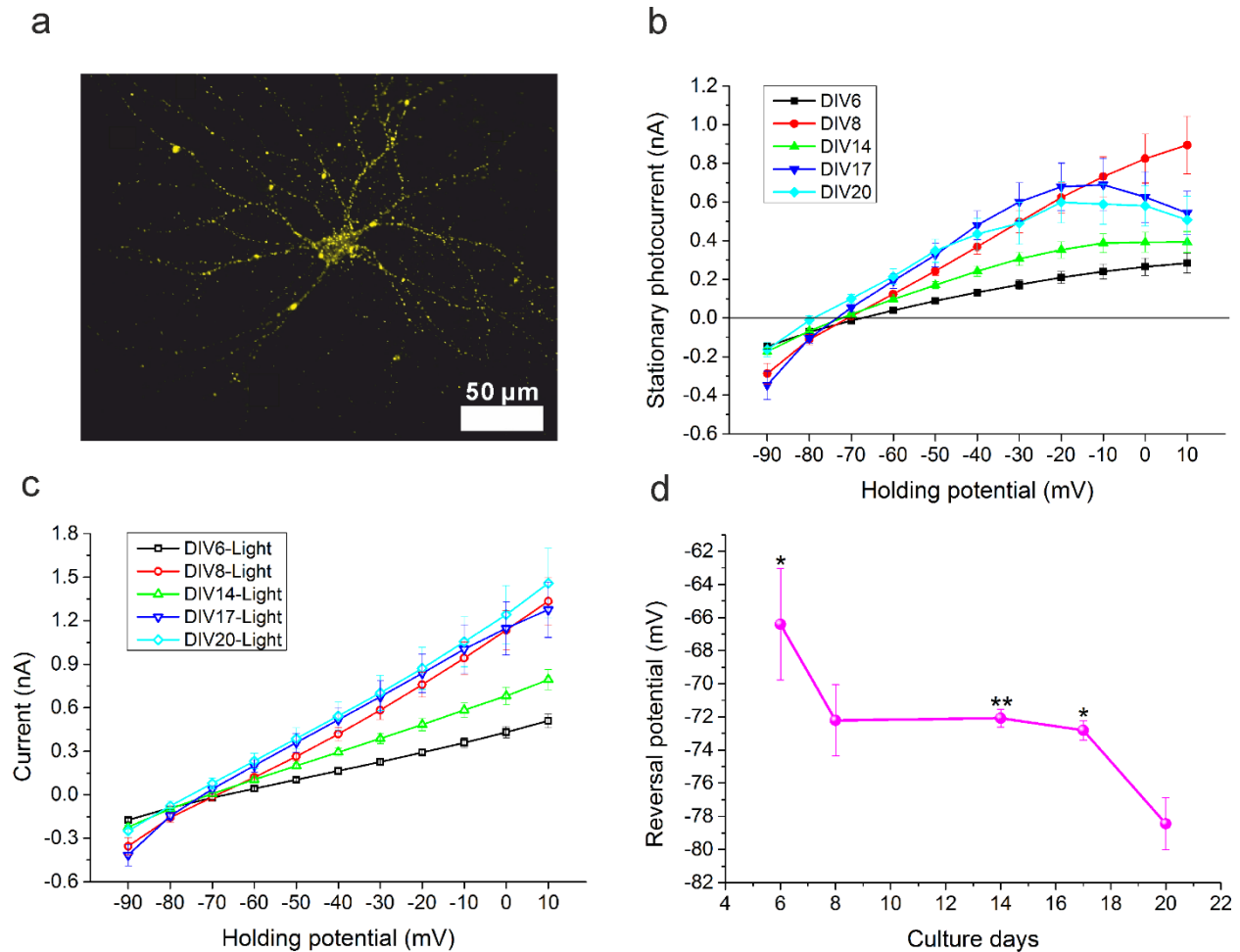
544 **Figure 2 Neurotransmission induced with ChR2-XXL**

545 a) Expression of ChR2-XXL-YFP in a primary rat cortical neuron on DIV13. b) Schematic of a
546 neurotransmission measurement. Patch clamp was employed to measure the response of the
547 ChR2-XXL negative neuron (the putative postsynaptic neuron) when the 473 nm laser
548 illuminated the putative presynaptic neuron that expressed ChR2-XXL. ChR2-XXL positive
549 neurons were identified by their fluorescence when selecting neuron pairs. c) Current traces
550 show peak currents of ChR2-XXL negative neurons during laser stimulation at ChR2-XXL
551 positive neurons (Holding potential: -70 mV). Blue bands indicate the period of laser stimulation.
552 d) Spiking associated with neurotransmission from the putative presynaptic neuron under
553 different durations of laser illumination. All traces were measured from the same neuron.
554 Injected holding current was -27 pA (laser illumination 1 second) or -19 pA for others. e) The
555 time of spiking evoked by the laser light of different durations ($n = 11, 9, 15$ and 8 cells for 1 sec,
556 10 sec, 30 sec, and 60 sec, respectively; one-way ANOVA, * $p = 0.0127255$, ** $p = 0.00107$, ***
557 $p = 0.000987$ vs. 1 sec group).



559 **Figure 3 Depolarisation block induced by ChR2-XXL**

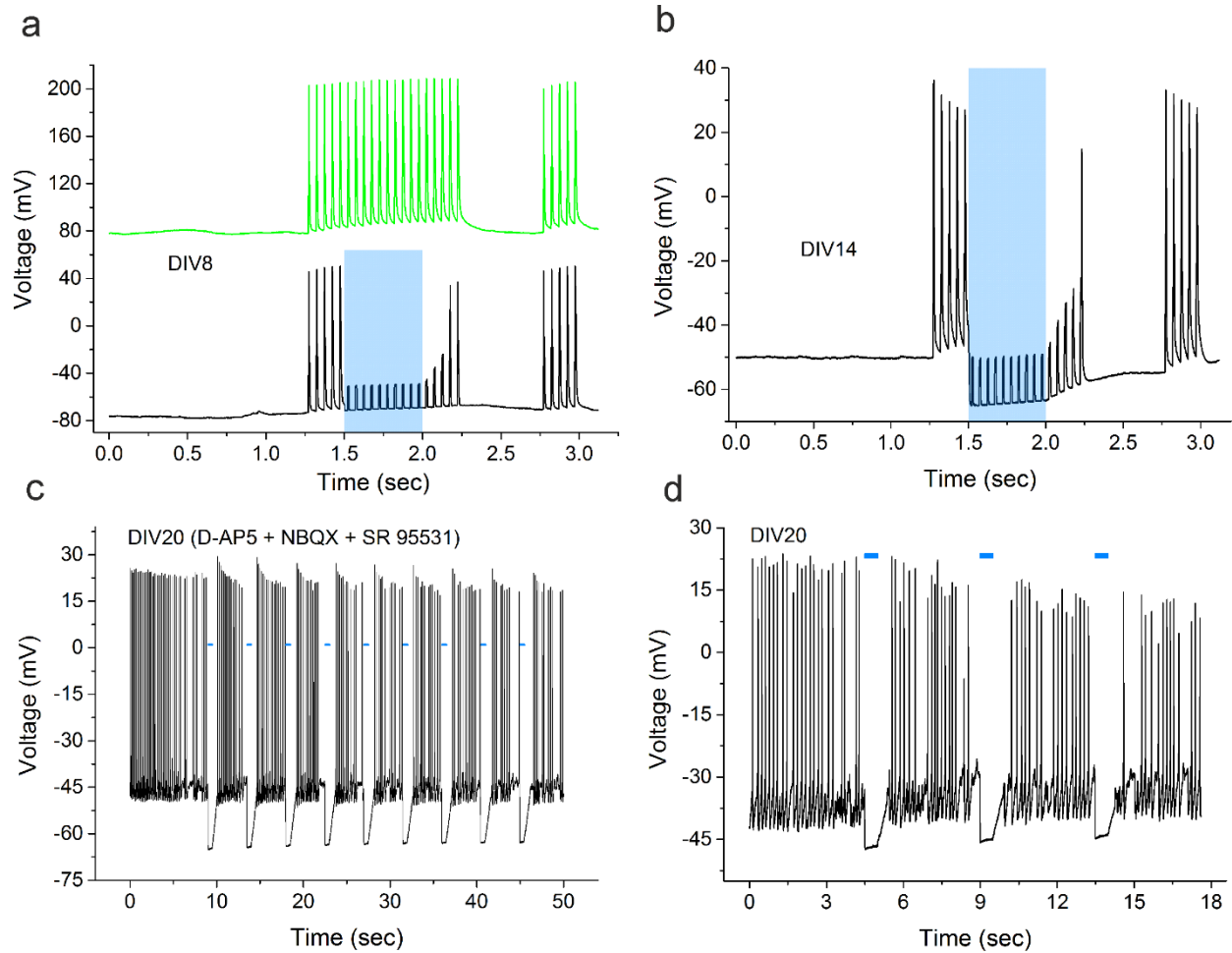
560 a) After 10 minutes exposure to white light, ChR2-XXL positive neurons (red unfilled circles)
561 needed more injected current than control neurons (ChR2-XXL negative neurons, black unfilled
562 circles) to hold the membrane potential at -70 mV (DIV7 to DIV17). b) The resting potential (0
563 pA injection) of ChR2-XXL positive neurons (red filled circles) was higher than control cells
564 (ChR2-XXL negative neurons, black filled circles) on DIV7 and DIV14. In both a) and b), each
565 circle is the average value over 1 second for one neuron (t-Test, ****: a) $p = 5.5233E-06$, b) $p =$
566 $2.72385E-10$). c) No action potentials of ChR2-XXL(+) neurons are induced by laser light when
567 directly targeted. Membrane potentials were measured with current clamp at DIV7 (laser
568 stimulation 1 sec, injected current: -16 pA), DIV10 (laser stimulation 1 sec, injected current: -190
569 pA; laser stimulation 30 secs, injected current: -170 pA), DIV14 (laser stimulation 1 sec, injected
570 current: -173 pA). d) Schematic of hyperpolarisation protocol to recover from depolarisation
571 block. Voltage steps from -70 mV to -20 mV in 10 mV increments were applied before and after
572 hyperpolarisation. Hyperpolarisation with different voltages and durations is indicated by the pink
573 region e) Recovery of ChR2-XXL positive neurons was not achieved by hyperpolarisation to -
574 130 mV for 5 secs. A typical current trace of ChR2-XXL positive neurons is shown in red, while
575 that of a non-transfected neuron is shown in black. F) Recovery of ChR2-XXL positive neurons
576 was successfully achieved by hyperpolarisation at the severe conditions of -170 mV for 2 secs
577 (Blue) and -210 mV for 2 secs (Orange). These traces are from a different neurons than **Fig. 2e**.



578

579 **Figure 4 IE relationship for GtACR1 in primary rat cortical neurons**

580 a) Yellow fluorescence is shown in a typical rat cortical neuron (DIV16) transfected with
581 GtACR1-YFP. b) IE relationship for GtACR1 in primary rat cortical neurons at different days. The
582 data (mean values \pm s.e.m.; $n = 3$ to 5 neurons) were baseline corrected by subtracting the
583 current from the last 50 milliseconds without light from the current of the last 50 milliseconds with
584 light. c) The current of GtACR1-expressing neurons clamped at different voltages from -90 mV to
585 10 mV and exposed to blue light. The data indicates mean values of the last 50 milliseconds \pm
586 s.e.m. ($n = 3$ to 5 neurons). d) Reversal potential for GtACR1 during neural development. The
587 reversal potential was defined as the point where the stationary photocurrent amplitude was 0
588 pA. At different days *in vitro*, the reversal potentials of GtACR1 (Mean values \pm s.e.m.; $n = 3$ to 5
589 cells) were determined by linear fits of the IE relationship curves as depicted in **Fig. 4b**. Data
590 were analysed by one-way ANOVA (* $p = 0.017878$ DIV6 vs. DIV20 group, $p = 0.032382$ DIV17
591 vs. DIV20 group, ** $p = 0.00858$ DIV14 vs. DIV20 group).



592

593 **Figure 5 Photoinhibition of primary rat cortical neural activity at different culture days**

594 a) Photoinhibition of spiking induced by pulsed current (300 pA for 10 milliseconds in one pulse,
595 20 Hz) in a typical GtACR1-expressing neuron of DIV8. Green trace indicates the
596 measurement without light (top), black trace (bottom) indicates the measurement with light for
597 500 msec (blue box). b) Photoinhibition of spiking induced by pulsed current (300 pA for 10
598 milliseconds in one pulse, 20 Hz) in a typical GtACR1-expressing neuron at DIV14. c) The
599 amplitude of action potentials increased after photoinhibition. In a typical neuron (DIV20)
600 expressing GtACR1, a continuous current of 100 pA was clamped near threshold so it
601 continually fired APs. Synaptic blockers were used in the bath. Nine blue laser pulses were
602 applied for inhibiting neural activity, each pulse lasted for 500 milliseconds separated by a delay
603 time of 4 seconds and showed higher amplitude APs when the firing resumed in the dark. d)
604 Photoinhibition of spontaneous activity in a GtACR1-expressing neuron at DIV20 without any
605 current injection or synaptic blockers. Each pulse of blue laser lasts 500 msec (blue bar). In all
606 measurements, blue laser intensity was 0.21 W/mm².